EXHIBIT I

TO DECLARATION OF SCOTT D. TANNER, PHD.

U.S. Patent Application Ser. No. 10/614,115

Simultaneous determination of proteins using an element-tagged immunoassay coupled with ICP-MS detection

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We report a novel method that suggests that multiple proteins can be quantified simultaneously in complex biological samples. The use of distinguishable element-tagged antibodies allows discriminant detection with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS), providing a sensitive and accurate means of determining the concentrations of specific proteins in complex mixtures. It is demonstrated that this method can be used to detect the levels of at least two target proteins at the same time, yielding a linear response to both proteins in a concentration range of 2–100 ng mL^{-1} using a sample size of 0.5 mL.

Introduction

The challenge of proteomics is to determine how proteins interact in vivo, with the goal of determining a means (i.e., using small molecule drugs) to control the pathway to disease. The analytical challenge is to provide sufficient sensitivity to determine the proteins at endogenous levels, while remaining largely oblivious to the bulk protein and salt matrix, and while providing quantitative information on the interaction stoichiometry. This challenge is facilitated through the use of affinity separation, which is specific for the target proteins and allows the isolation of target species. A common protocol is to grow cell cultures under near-natural conditions, with or without an agonist, and harvest the proteins following lysing of the cell by fishing with target-specific antibodies. Often, the cell is first transfected so that the target proteins are labeled with an organic moiety that has the desired affinity for the antibodies used. In its most common form, immunoassays employ fluorescent, enzymatic or radiological tags for detection.

An immunoassay can be described as the detection and quantitation of a specific protein (antigen) with complementary antibodies. The strong affinity of antigen–antibody interactions forms largely through non-covalent bonds and conformational fit. The high specificity and reactivity of the antigen–antibody interaction allows for many biotechniques, including: (i) the separation and purification of antigens of interest through immobilized complimentary antibodies; (ii) the visualization of specific cellular proteins and structures through fluorescent-conjugated antibodies, and (iii) immunoassays or the quantitation of a particular antigen through enzyme- or fluorescent-linked antibodies (in which the outcome of an enzymatic or fluorometric reaction is proportional to the amount of antigen present). There are many methods of tagging biologically active materials that can provide analysis using different physical methods of detection. The recent development of metal-tagged antibodies, for purposes other than element detection, makes possible the involvement of atomic spectroscopy in these biotechniques.

ICP-MS has analytical characteristics that are complementary to the conventional protocols. Of special merit are the sensitivity, large dynamic range, independence of the sample matrix and large number of elements and isotopes that can be simultaneously determined. Combining these attributes with the specificity of immunoreaction offers a new approach to the proteomic challenge. The premise of ICP-MS-linked immunoassays is straightforward. Antigens of interest are reacted with complementary, metal-tagged antibodies, physically separated from non-reacting proteins, and then the atomic composition of the tag conjugated to the antibody is measured to determine the antigen concentration of the sample. Clearly, the sensitivity of the method is a linear function of the number of atoms of a given isotope in the tag. It is also clear that accurate quantitation demands that the number of atoms in similar tags has a narrow distribution. Further, multiple antibodies can be labeled with distinguishable element tags (as elements, isotopes or in unique combinations; preferably those that occur at naturally low levels), potentially allowing simultaneous determination of multiple antigens provided that the immunochemical conditions are favourable and the reactions are independent.

As tags have not yet been developed specifically for elemental analysis, we have made use of immuno-reagents that already contain element tags (e.g., gold and lanthanides) for different purposes. For example, gold-tagged antibodies and ligands are routinely used in the localization of cellular proteins using colloidal gold in electron microscopy[1–5] and have been successfully analyzed by ICP-MS.[6–11] These tags contain either colloidal gold or small gold clusters (less than 2 nm in diameter). This is an advantage when using ICP-MS for detection, as the elemental nano-particles used are of uniform size and contain a significant number of atoms per conjugate. In addition, there is also the option of increasing the Au signal response even further by using silver enhancement.[5]

To the unanticipated advantage of atomic spectrometry, four lanthanide (Eu, Tb, Dy, and Sm) tags have been conjugated to various biologically active materials and are marketed for use in an automated fluoroimmunometric system (Wallac AutoDELFIA[12]). In these assays, lanthanide-labeled antibodies (or competing peptides) are reacted with the antigen of interest in an ELISA (Enzyme Linked ImmunoSorbent Assay) and then quantified by the addition of chelators, which release the lanthanide ions to form fluorescent chelates.

We have previously described several novel ICP-MS-linked immunoassays using both nanogold and lanthanide-tagged antibodies, in which some commonly-used immunoaffinity separation techniques (centrifugal filtration, gel filtration, protein A sepharose affinity, and ELISA) were successfully coupled to the ICP-MS to detect and accurately quantify the specific

concentrations of target proteins in complex biological samples.[7,8] The benefits of elemental detection over fluorescence include: (i) elimination of chelator step, (ii) multi-analyte detection (e.g. elemental analysis provides the possibility of detecting multiple antigens per sample), and (iii) long-term sample integrity.[8]

"Real life" immunoassays are performed conveniently in 96-well plate format employing a specific well coating. In this report, we demonstrate a useful and important immunosorbent method using maleic anhydride plates[13] linked to ICP-MS. In this format, two protein targets were analyzed simultaneously in each sample. This brings to light future possibilities of quantitatively determining protein–protein interactions and the stoichiometry of biological complexes.

Experimental

Apparatus and materials

Experimental measurements of elemental compositions were made on a commercial quadrupole mass spectrometer, ELAN 6100 ICP-MS (Perkin Elmer SCIEX) that has been previously described.[14] The operating conditions are summarized in Table 1. In all experiments, the sample uptake was directed through a MicroFlow PFA-ST concentric nebulizer (Elemental Scientific, Inc.) using an autosampler (Perkin Elmer AS 91) modified for sampling from 96 deep well microtiter plates.

Reagents included: NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 µg mL^{-1} leupeptin, 1 µg mL^{-1} aprotinin, and 1 µg mL^{-1} pepstatin), 1 × phosphate buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4), 1–3% bovine serum albumin (BSA) in PBS (1–3% BSA), TBST (25 mM Tris, 0.15 M NaCl, pH 7.2, 0.05% Tween-20), Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% fetal bovine serum (Gibco) in DMEM (10% FBS), 5% horse serum (Gibco) in DMEM (5% HS), 10% (v/v) HCl (Seastar Chemical Inc.), protein A Sepharose CL-4B (Amersham Pharmacia), human IgG (AG722 Chemicon Lot 20031151), NANOGOLD goat anti-human Fab' conjugate [αh-Fab'(g)Au; #2053, Nanoprobes], NANOGOLD goat anti-rabbit Fab' conjugate [αr-Fab'(g)Au; #2004, Nanoprobes], NANOGOLD goat anti-mouse Fab' conjugate [αm-Fab'(g)Au; #2002, Nanoprobes], Eu-labeled monoclonal anti-FLAG conjugate [αFLAG(m)Eu; #AD0056, Wallac/Perkin Elmer], anti-Smad2/3 rabbit polyclonal IgG [αSmad2(r), #06-654, Upstate Biotechnology], anti-Smad2 monoclonal IgG [αSmad2(m), S66220], anti-Smad4 monoclonal IgG [αSmad4(m), S71120, BD Transduction Laboratories], anti-phospho-Smad2 rabbit polyclonal IgG [αPSmad2(r), #06-829, Upstate Biotechnology], goat anti-mouse HRP conjugate [αm-HRP, sc-2005, Santa Cruz], goat anti-rabbit HRP conjugate [αr-HRP, sc-2004, Santa Cruz], N-terminal 3 × FLAG-BAP control protein (3 × FLAG-BAP; P-2104, Sigma), GST-Smad2 bacterial expression vector [full length clone of Smad2 (1–467 amino acids) inserted into the pGEX4T-1 vector (Pharmacia)], ECL Super Signal (Pierce), BCA reagent (Pierce), REACTI-BIND maleic anhydride activated polystyrene plates (#15100, Pierce), Ir and Ho diluted from stock 1000 ppm solutions (SPEX) to 1 ppb in

10% (v/v) HCl, Masterblock 96 deep well microtiter plates (Greiner). All solutions were prepared using de-ionized (Elix/Gradient water purification system, Millipore) distilled water.

The NANOGOLD Fab'-nanoAu reagents consist of affinity purified Fab' fragments covalently bound to 1.4 nm nanogold clusters. The Eu-labeled conjugates consist of approximately 6–10 atoms of Eu tagged to the antibody reagents and affinity purified.

Results and discussion

Multi-analyte ICP-MS-linked immunoassay

In these experiments, we make use of two sets of element-tagged antibodies: Fab'-nanoAu fragments and Eu-labeled whole molecule antibodies. One potential disadvantage of using gold in this manner is that gold has a high affinity for surfaces of a typical ICP-MS sample introduction system. However, it was observed that this effect is significantly reduced in the presence of proteins in a sample, probably due to complexation and/or passivation of surfaces.[8] The concentrations of commercially available Fab'-nanoAu is not specified by the manufacturer as conventional applications do not require absolute quantitation. However, the manufacturer does specify that each gold cluster is approximately 70 atoms. The elemental composition of the Fab'-nanoAu reagents has been previously characterized by ICP-MS.[8] We have found that although chromatographically purified by the manufacturer, approximately 50–60% of the gold signal is not associated with the Fab'-nanoAu, although this result is obviously lot-dependent. We expect that these nanoAu impurities have minimal impact on the results since they are easily removed during separation and washing procedures.

Iridium is used as an internal standard for gold because gold and iridium have close atomic mass numbers and their ionization potentials are similar (9.225 and 9.1 eV, respectively). Therefore, the instrument response is expected to be comparable for both analytes using an ICP ion source. In addition, both Au and Ir are stable in HCl, which is a useful medium for dissolving protein samples. In this work, the ratio of the ion signals, ^{197}Au/(^{191}Ir and ^{193}Ir) in a standard solution containing 1% BSA acidified (1 : 1) with 10% (v/v) HCl, 0.1% HF, 1 ppb Au and 1 ppb Ir was used to quantify the gold content in experimental samples.

The Eu-labeled IgG molecules contain approximately 6–10 atoms of Eu per IgG molecule as specified by the manufacturer. Eu has two isotopes of similar abundance, and we therefore expect that 3–5 atoms of each Eu isotope (^{151}Eu, 47.8% abundant and ^{153}Eu, 52.2% abundant) exist per tag. Holmium was chosen as an internal standard for europium measurements since europium and holmium have close atomic mass numbers and their ionization potentials are similar (5.67 and 6.02 eV, respectively).

ICP-MS-linked maleylation immunoassay

The ICP-MS-linked maleylation immunoassay was developed to quantitate multiple proteins simultaneously. This assay takes advantage of commercially available REACTI-BIND maleic anhydride polystyrene 96 well plates, which use reaction chemistry to anchor proteins to the bottom of 96 well plates. The reaction (maleylation) between maleic anhydride with the N-terminal and lysine amine groups of proteins results in the formation of an amide bond that is stable at neutral pH. These plates were developed for ELISAs, in which proteins in a biological solution could be anchored to the bottom of the plate and could then be detected using a primary antibody raised against the antigen of interest, a secondary antibody conjugated with horse radish peroxidase (HRP), and a substrate

Table 1 ICP operating conditions

Sample introduction	Peristaltic pump (from 20 µL to 0.5 mL min^{-1} uptake)
	MicroFlow PFA-ST concentric nebulizer (ESI)
	Quartz cyclonic spray chamber
RF plasma source	Free-running (nominal 40 MHz) ICP 1400 W (typical)
Plasma gas	15 L min^{-1} Ar
Auxiliary gas	1.2 L min^{-1} Ar
Nebulizer gas	1.02 L min^{-1} Ar (optimized for Ir)

for HRP. The outcome of the enzymatic reaction is measured allowing for the estimation of antigen concentration.[13]

In the following sets of experiments, we have used maleic anhydride plates coupled to the ICP-MS to determine accurate concentrations of specific antigens based on elemental analysis. It is an attractive method for an immunoassay because of its simplicity, speed of analysis and its straightforward automation. We compare this method to traditional Western blotting and in the following experiment, five cell lysates were harvested and two target antigens (endogenous proteins Smad2 and Smad4) were detected by both methods. Smad2 is a protein which is activated through phosphorylation. These antigens were chosen to test the sensitivity of ICP-MS-linked immunoassay detection of rare proteins in cellular complex mixtures. Both Smad2 and Smad4 are transcriptional mediators that exist at low levels in many cell types (including the mouse skeletal muscle C2C12 cell line). In this experiment, C2C12 cell lines were plated on 1% gelatin and grown in 10% FBS in 100 mm plates. After 2 days, the medium was replaced with 5% HS to induce myogenesis. Subsequently, the medium was replaced every 2 days with 5% HS for 12 days. In this way, the protein levels of Smad2 and Smad4, and the rate of phosphorylation of Smad2 could be monitored over a time line of 2–12 days during myogenesis. It is important to note that these cells were not exposed to exogenous TGF-β (transforming growth factor beta), which is known to cause the phosphorylation of Smad2. It is therefore thought that a very small percentage of Smad2 would be phosphorylated at any time. Cells were harvested after 2 days, 4 days, 6 days, 8 days, and 12 days in NP-40 lysis buffer and stored at −20 °C. A BCA protein assay using BCA reagent was performed on each of the five lysates to determine the total protein concentrations.

The ICP-MS maleylation immunoassay was performed in which 100 µL of 100 µg of each lysate was incubated in wells of maleic anhydride plates for 1 h at room temperature to allow maleylation to occur. Each sample was prepared in triplicate wells. The wells were then blocked with 3% BSA for 1 h and then probed with primary antibody (e.g. either monoclonal αSmad2, monoclonal αSmad4, or rabbit αPSmad2 as indicated) for an hour at room temperature. After washing with 3% BSA,

a secondary antibody [αm-Fab'(g)Au or αr-Fab'(g)Au] was added and incubated for 1 h. The wells were washed 3 times, dried, and then acidified with 10% HCl spiked with 1 ppb Ir. Negative controls (100 µL of lysis buffer per well for each antigen detected) were probed in the same manner with both primary and secondary antibodies. A calibration curve of affinity purified GST-Smad2 over two orders of magnitude (2.08 to 208 ng per well) was used to determine the concentration of Smad2 in the cell lysates and estimate the concentration of Smad4 and phosphorylated Smad2 (Fig. 1). In this case, we have assumed that GST-Smad2 has the same affinity for monoclonal α-Smad2 as endogenous Smad2 does. Ideally, purified endogenous Smad2 would serve as a calibration control. We found that the concentrations of Smad2 and Smad4 and the levels of Smad2 phosphorylation do not change significantly during myogenesis. Over the time course, the levels of Smad2 were approximately 12.5 ± 2.8 ng Smad2 protein per 100 µg lysate.

In a comparison study, we quantified the Smad2, Smad4 and phosphorylated Smad2 levels of these five lysates using conventional Western blotting and densitometry. 100 µg of each cell lysate was loaded onto a 7.5% SDS-polyacrylamide gel and run at 200 V for 1 h. The gel was blotted onto nitrocellulose and the blot was blocked with TBST for 1 h. The blot was probed with a primary antibody (i.e. α-Smad2, α-Smad4, or α-PSmad2 depending on the target antigen), washed with TBST, exposed to an anti-mouse-HRP antibody, and washed again. A chemiluminescent substrate (ECL Super Signal) was added and the blot was exposed for 20 s on an imager to quantify the relative densities of the protein bands on the blot. Negative controls consisted of random background density measurements on each blot. For calibration, 5, 50, and 100 ng of unreduced primary antibodies (i.e. α-Smad2, α-Smad4, or α-PSmad2 as indicated) were loaded onto the first 3 lanes of each gel. It is assumed that when probing the blot with a secondary antibody, its affinity for the primary is similar to its affinity for the primary–antigen complex. The results of the densitometry are shown in Fig. 2 A–C.

We have previously found that the relative densities of the blots do not accurately reflect protein concentration over a
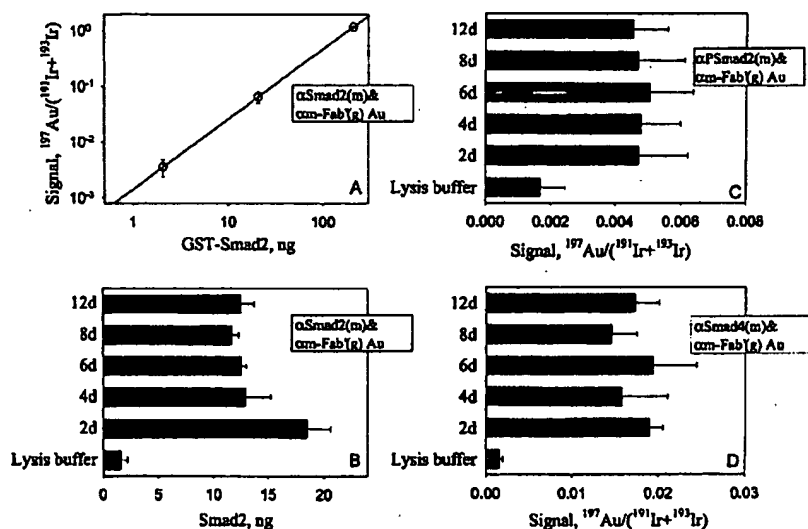

Fig. 1 ICP-MS-linked maleylation immunoassay of cultured cell lysates. (A) A calibration curve of affinity purified GST-Smad2 was used to determine the concentration of Smad2 (B). (B–D) Five C2C12 cell lysates were harvested at 2, 4, 6, 8, and 12 days(d). 100 µL of each lysate (1 mg mL⁻¹) was immobilized in triplicate in maleic anhydride plates and probed with both primary [i.e. (B) αSmad2(m), (C) αPSmad2(r), (D) αSmad4(m)] and NANOGOLD-tagged secondary antibodies [i.e. (B) αm-Fab'(g)Au, (C) αr-Fab'(g)Au, and (D) αm-Fab'(g)Au]. The ICP signal is background subtracted.

Density, mean value CNT

Smad2 — A

Smad4 — B

PSmad2 — C
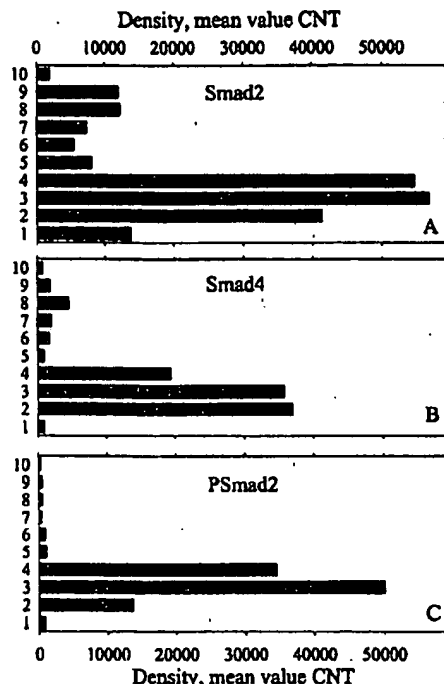
Density, mean value CNT

Fig. 2 Western blotting of cultured cell lysates. (A–C). Five C2C12 cell lysates were harvested at 2, 4, 6, 8, and 12 days (bars 5–9, respectively; bar 10 is the negative control). 100 µg of each cell lysate was analyzed using SDS-PAGE, blotting on nitrocellulose, probing with a primary antibody [i.e. (A) αSmad2(m), (B) αSmad4(m), (C) αPSmad2(r)], and HRP-tagged secondary antibody [i.e. (A and B) αm-HRP, (C) αr-HRP]. The relative densities (CNT, counts) of 5, 50, and 100 ng (bars 1, 2 and 3, respectively) of primary antibody [i.e. (A) αSmad2(m), (B) αSmad4(m), (C) - αPSmad2(r)] were measured and used to calculate the average protein concentrations of Smad2, Smad4, and PSmad2. A positive control was included in each gel (bar 4). (A) 50 ng GST-Smad2, (B) 50 ng mouse IgG, (C) 50 ng rabbit IgG.
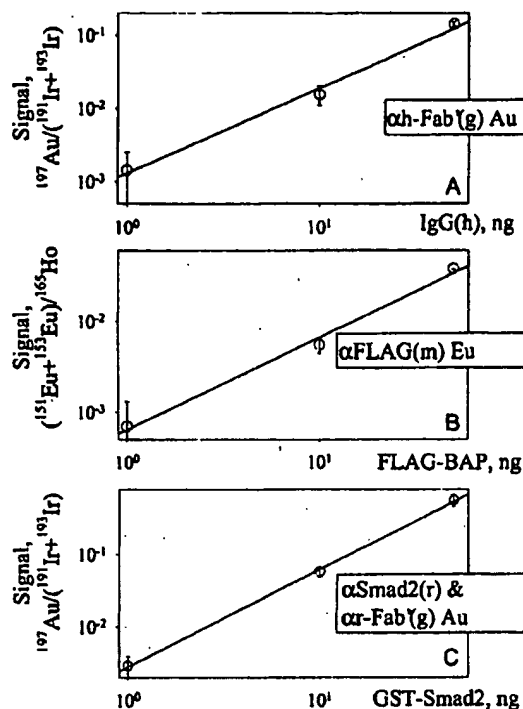

Fig. 3 Detection of multiple proteins using ICP-MS-linked maleylation immunoassay. (A–C) Three purified proteins (human IgG, 3 × FLAG-BAP, and GST-Smad2) were immobilized on maleic anhydride plates in concentration ranges of 1–50 ng per sample. The antigens were detected by probing with primary antibody [i.e. (A) αh-Fab'(r)Au, (B) αFLAG(m)Eu, and (C) αSmad2(r)] and secondary antibody [(C) αr-Fab'(g)Au]. Each sample was prepared in triplicate and the standard error was calculated based on the average.

the DL of the ICP-MS maleylation immunoassay would be improved.

range of concentrations.[8] The expected linear relationship between density and protein concentration is usually observed over one order of magnitude, which might be attributed to the limited capability of the imager in data quantitation. This result can be seen again in the calibration curves of each of the three Western blots (bars 1–3, Fig. 2A; bars 1–3, Fig. 2B; bars 1–3 Fig. 2C). By plotting a linear regression only for 0, 5 and 50 ng of antigen, we estimated the concentrations of Smad2, Smad4 and phosphorylated Smad2. Again, we found that the concentrations of Smad2 and Smad4 and the levels of Smad2 phosphorylation do not change significantly over this time course. The protein levels of Smad2, Smad4, and phosphorylated Smad2 average at 12.3 ± 5.2, 2.1 ± 1.5, and 3.3 ± 2.0 ng per 100 µg lysate respectively. The results using Western blot density analysis have a greater error associated with them compared to the previous ICP-MS-linked immunoassay (Fig. 1B; 12.5 ± 2.8 ng Smad2 protein per 100 µg lysate), but fall into the same protein concentration range expected for these proteins.

However, despite the limited quantitative abilities, Western blotting does have one major advantage over the ICP-MS-linked maleylation immunoassay in that all the samples were loaded in a volume of 20 µL. In the ICP-MS-linked assays, we kept the sample volume to 0.5 mL, which was convenient for the standard sample introduction system, with an aspiration rate of approximately 100 µL min^{-1}. If the sample introduction system and uptake rate could be reduced,

Multi-analyte detection using ICP-MS-linked maleylation immunoassay

In this experiment, ICP-MS-linked maleylation immunoassays were used to detect and quantitate two antigens simultaneously. Three purified proteins (Human IgG, 3 × FLAG-BAP, and GST-Smad2) were used as target antigens singly (Fig. 3) or in combination (Fig. 4) in concentration ranges of 1–50 ng per 100 µL. Again, we have used maleic anhydride plates coupled to the ICP-MS to determine accurate concentrations of the specific antigens. The protein concentration of purified GST-Smad2 was determined using a BCA protein assay. Serial dilutions (1, 10, 50 ng per 100 µL per well) of each of the 3 purified proteins were prepared in PBS and incubated for an hour in wells of maleic anhydride plates to allow maleylation to occur. Negative controls for the experiment consisted of 100 µL of PBS without protein. Each sample was prepared in triplicate wells. The wells were then blocked with 3% BSA for 1 h and probed with primary antibody [e.g. αh-Fab'(r)Au, αFLAG(m)Eu, and/or αSmad2(r) as indicated] for an hour at room temperature. Wells containing GST-Smad2 were also probed with secondary antibody [αr-Fab'(g)Au]. Wells were washed 3 times (with 3% BSA, 0.05% Tween-20) between incubations. After the final wash, the wells were dried and acidified with 10% HCl spiked with 1 ppb Ir. We found that the detection of each protein through element-tagged antibodies gave a linear response of signal (plotted as cps/cps; analyte/1 ppb spike; i.e. Au/Ir, Eu/Ho) to antigen concentration with a
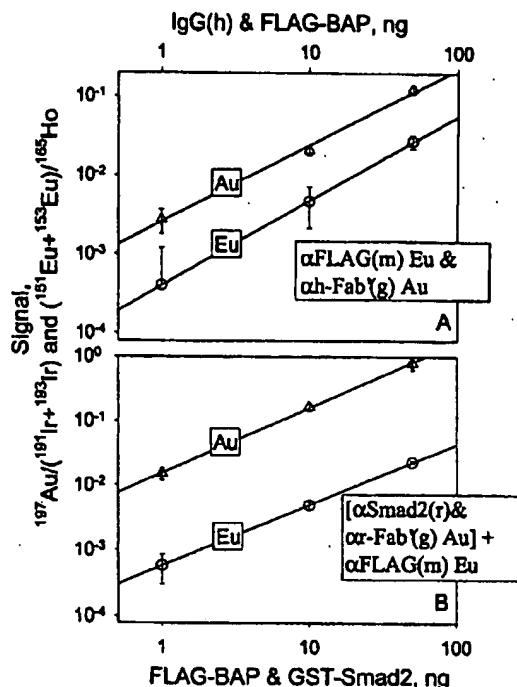
Fig. 4 Simultaneous detection of multiple proteins using ICP-MS-linked maleylation immunoassay. (A and B). ICP-MS-linked maleylation immunoassays were used to detect and quantitate three antigens (e.g. human IgG, 3 × FLAG-BAP, and GST-Smad2) in combination. Maleic anhydride plates were used to immobilize the antigens, which were probed with primary antibody [i.e. (A) αh-Fab'(r)Au and αFLAG(m)Eu, (B) αFLAG(m)Eu and αSmad2(r)] and secondary antibody [i.e. (B) αr-Fab'(g)Au]. Each sample was prepared in triplicate and the standard error was calculated.

low standard deviation between triplicate samples (Fig. 3 A–C). The detection of 2 antigens simultaneously was equally successful, with similar signal responses (of the metal tag analyte, cps) to concentration, low background, and low standard of deviation between triplicate samples. The results are presented in Fig. 4 A–B.

Conclusions

We have demonstrated that a novel ICP-MS-linked immunoassay, in which maleic anhydride plate immunoreactions are coupled to ICP-MS detection can be used to measure at least two antigens simultaneously. It is apparent that this method has broad multi-analyte capabilities through which many different antigens as well as protein–protein interactions can be quantitatively measured through elemental analysis, provided that distinguishable element tags are used.

In addition to the well known advantages of ICP-MS as an elemental detector, ICP-MS-linked immunoassays offer significant benefits over traditional methods, such as: (i) the analysis of the tag is performed directly, eliminating the need for a substrate or chelator, (ii) biological impurities or contaminants do not affect elemental analysis results, (iii) non-specific background is not a function of time, unlike ELISAs in which the background depends on the incubation time, (iv) immediate acidification of the reacted sample allows for long-term storage before analysis, and (v) detection limits are improved linearly with multiple tagging of a single isotope.

References

1   M. Bendayan, Science, 2001, 291, 1363–1365.
2   J. F. Hainfeld and F. R. Furuya, J. Histochem. Cytochem., 1992, 40, 177–184.
3   J. F. Hainfeld and R. D. Powell, J. Histochem. Cytochem., 2000, 48, 471–480.
4   J. F. Hainfeld and J. M. Robinson, J. Histochem. Cytochem., 2000, 48, 459–460.
5   Nanogold Conjugates, Nanoprobes, http://www.nanoprobes.com
6   E. J. Andreu, J. J. Martin de Llano, I. Moreno and E. Knecht, J. Histochem. Cytochem., 1998, 46, 1199–1201.
7   V. I. Baranov, D. R. Bandura and S. D. Tanner, presented at the European Winter Conference on Plasma Spectrochemistry, Hafjell, Norway, 2001, Book of Abstracts, p.85.
8   V. I. Baranov, Z. A. Quinn, D. R. Bandura and S. D. Tanner, Anal. Chem., 2002, 74(7), 1629–1636.
9   J. J. Martin de Llano, E. J. Andreu and E. Knecht, Anal. Biochem., 1996, 243, 210–217.
10 J. J. Martin de Llano, E. J. Andreu, A. Pastor, M. de la Guardia and E. Knecht, Anal. Chem., 2000, 72, 2406–2413.
11 C. Zhang, Z. Zhang, B. Yu, J. Shi and X. Zhang, Anal. Chem., 2002, 74, 96–99.
12 DELFIA, Perkin Elmer, Life Sciences, http://www.perkinelmer.com
13 Pierce, Maleic Anhydride Plates, http://www.piercenet.com/
14 S. D. Tanner, J. Anal. At. Spectrom., 1995, 10, 905–921.